S-Nitrosylation at the active site decreases the ubiquitin-conjugating activity of ubiquitin-conjugating enzyme E2 D1 (UBE2D1), an ERAD-associated protein

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Abbreviations

NO: Nitric oxide, ER: Endoplasmic reticulum, PDI: Protein disulfide isomerase, IRE1: Inositol-requiring enzyme 1, ERAD: ER-associated degradation, SGK1: Serine/threonine-protein kinase 1, UBE2D1: Ubiquitin-conjugating enzyme E2 D1, PD: Parkinson's disease, AD: Alzheimer's disease, NOS: Nitric oxide synthase, NADPH: Nicotine dinucleotide phosphate, SNO: *S*-Nitrosylated, ATF6: Activating transcription factor 6, PERK: PKR-like ER kinase, HEK293T: Human embryonic kidney 293T, SNOC: *S*-Nitrosocysteine, GSNO: *S*-Nitrosoglutatione, LC-MS/MS: Liquid chromatography-tandem mass spectrometry, CHIP: C-terminus of Hsc70-interacting protein, ARPD: Autosomal recessive PD

Abstract

S-Nitrosylation of protein cysteine thiol is a post-translational modification mediated by nitric oxide (NO). The overproduction of NO causes nitrosative stress, which is known to induce endoplasmic reticulum (ER) stress. We previously reported that *S*-nitrosylation of protein disulfide isomerase (PDI) and the ER stress sensor inositol-requiring enzyme 1 (IRE1) decreases their enzymatic activities. However, it remains unclear whether nitrosative stress affects ER-associated degradation (ERAD), a separate ER stress regulatory system responsible for the degradation of substrates via the ubiquitin-proteasomal pathway.

In the present study, we found that the ubiquitination of a known ERAD substrate, serine/threonine-protein kinase 1 (SGK1), is attenuated by nitrosative stress. C-terminus of Hsc70-interacting protein (CHIP) together with ubiquitin-conjugating enzyme E2 D1 (UBE2D1) are involved in this modification. We detected that UBE2D1 is *S*-nitrosylated at its active site, Cys85 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, *in vitro* and cell-based experiments revealed that *S*-nitrosylated UBE2D1 has decreased ubiquitin-conjugating activity.

Our results suggested that nitrosative stress interferes with ERAD, leading to prolongation of ER stress by co-disruption of various pathways, including the molecular chaperone and ER stress sensor pathways. Given that nitrosative stress and ER stress are upregulated in the brains of patient with Parkinson's disease (PD) and of those with Alzheimer's disease (AD), our findings may provide further insights into the pathogenesis of these neurodegenerative disorders.

1. Introduction

NO is a gaseous molecule with a short half-life [1,2]. NO is generated by nitric oxide synthase (NOS), an enzyme that synthesizes NO from L-arginine using nicotine dinucleotide phosphate (NADPH) and oxygen. NO possesses a free radical moiety that reacts posttranslationally with the cysteine thiols of proteins to form *S*-nitrosothiol. Previous studies have reported that *S*-nitrosylated (SNO-) proteins exhibit altered enzymatic activity, localization, and/or structure [3,4]. Physiological levels of NO have been shown to regulate apoptosis, neurotransmission, and cell proliferation [5–7]. On the other hand, excessive amounts of NO cause nitrosative stress and contribute to the pathogenesis of various diseases, including neurodegenerative disorders, cancer, and diabetes[8–10].

How does nitrosative stress affect such diseases? The answer may come from the association of nitrosative stress with ER stress. ER stress is induced by the accumulation of unfolded proteins at the ER. During ER stress events, three different sensors, activating transcription factor 6 (ATF6), IRE1, and PKR-like ER kinase (PERK), are activated to deal with this stress. Following the activation of ER stress sensors, the molecular chaperone genes such as PDI are upregulated to help protein folding and ERAD finally to degrade unfolded protein by the ubiquitin-proteasome system. We previously reported that *S*-nitrosylation of PDI and IRE1 attenuates their enzymatic activities and induced severe ER stress[8,11].

These lines of evidence support that NO is the modulator for ER stress, however its effect on ERAD remains unclear. Here, we showed that the ubiquitination of a known ERAD substrate, SGK1, is attenuated by nitrosative stress. Further analysis revealed that the active site of ERAD-associated UBE2D1 is target for *S*-nitrosylation, which

may decrease ubiquitin-conjugating activity. Our findings will shed light on the unknown modulatory effect of NO on ERAD and this might be the key to understanding the pathogenesis of nitrosative stress-related disorders.

2. Material and methods

2.1 Reagents and antibodies

The *in vitro* ubiquitination assay was performed using the CHIP/Luciferase Ubiquitination Kit (Boston Biochem, K-280, Lot #16559217) and Human recombinant UBE2D1 (Boston Biochem, E2-616). The following antibodies were purchased from the indicated vendors: anti-Myc antibody (Cell Signaling Technology, #2276), anti-Akt antibody (Cell Signaling Technology, #9272S), anti-phospho-Akt (S473) (D9E) XP(R) antibody (Cell Signaling Technology, #4060), anti-GAPDH antibody (Cell Signaling Technology, #2118), anti-FLAG antibody (Sigma-Aldrich, A8592), anti-ubiquitin antibody (Sigma-Aldrich, MAB1510), anti-T7 antibody (MBL, PM022), anti-goat IgG-HRP (Santa Cruz Biotechnology, sc-2354), anti-mouse IgG-HRP (GE Healthcare, NA9310), and anti-rabbit IgG-HRP (GE Healthcare, NA9340).

2.2 Cell culture

Human embryonic kidney (HEK) 293T cells and were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air.

2.3 Construction of plasmids

The cDNAs encoding the UBE2D1 and SGK1 proteins were derived from the

Genome Network Project (RIKEN BRC) clones HGY088581 and HGX001612, respectively [12]. The *UBE2D1* cDNA was amplified by Tks Gflex™ DNA Polymerase (TaKaRa Bio, R060A) using the following primer pair: 5'-AAA GCG GCC GCG ATG GCG CT-3' and 5'-CCC GGG ATC CTT ACA TTG C-3'. The resulting PCR product was digested with BamHI and NotI and subcloned into the similarly digested p3×FLAG-CMV10 vector (Sigma-Aldrich, E7658). The *SGK1* cDNA was amplified by Tks Gflex™ DNA Polymerase (TaKaRa Bio, R060A) using the following primer pair: 5'-CCC CGG ATC CCC ACC ATG ACG GTG AAA ACT GAG-3' and 5'-GGG CTC GAG GAG GAA AGA GTC CGT GGG-3'. The resulting PCR product was digested with BamHI and XhoI and subcloned into the similarly digested pcDNA™6 myc-His vector (Thermo Fisher Scientific, V22120). The inserted sequences were confirmed by the Sanger sequencing.

2.4 MG132 and LY294002 treatment

HEK293T cells were transfected pcDNATM6-myc-His SGK1. At 24hr after transfection, cells were treated with the proteasome inhibitor MG132 (CEM, CS-0471; at 5 or 25 μM) or the autophagy inhibitor LY294002 (CAY, 70920; 5 or 10 μM) for 3 hr.

2.5 Immunoprecipitation

To examine the ubiquitination levels of SGK1, HEK293T cells transfected with the myc-SGK1 were solubilized using Immunoprecipitation buffer (phosphate-buffered saline (PBS) containing 1% NP-40, 0.5% deoxycholic acid sodium salt, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM N-

ethylmaleimide, 1 mM NaF, and protease inhibitor cocktail (Roche)) and centrifuged at 20400 × *g* for 10 min at 4°C. The supernatant then was treated with an anti-Myc antibody, and bond proteins were immunoprecipitated by binding to Protein G Sepharose 4 Fast Flow beads (GE Healthcare, 17061801) prepared as follow. In brief, the beads were pretreated with 1% bovine serum albumin (BSA) for 1 hr to block non-specific binding, and then washed three times with Neutralization buffer (20 mM HEPES (pH 7.7), 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). The blocked beads were combined with cell lysates and incubated overnight at 4°C. The beads then were recovered and washed five times with Neutralization Buffer. Bound proteins were eluted from the beads using Laemmli sample buffer (62.5mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS) and analyzed by western blotting.

2.6 Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton-X, 0.1% SDS, and 1% sodium deoxycholate supplemented with protease inhibitor cocktail (Roche)) or Immunoprecipitation buffer. Each sample was boiled in Laemmli buffer and analyzed by western blotting as described previously [13]. The quantification analysis of [Ub]n-SGK1, SNO-UBE2D1, and [Ub]n-Luciferase was performed using Image J software (NIH, version 1.51j8).

2.7 Biotin-switch assay

SNOC-exposed HEK293T cells were subjected to the biotin-switch assay, as previously described [13]. Briefly, cells were lysed in ice-cold HEN buffer (250 mM HEPES-NaOH (pH 7.7), 1 mM EDTA, 0.1 mM neocuproine) containing 1% Triton X-

100 and protease inhibitor cocktail (Roche). Aliquots (800 μg total protein each) were subjected to the biotin-switch assay. Samples were stirred with blocking buffer (9 volumes of HEN buffer plus 1 volume 25% SDS, adjusted to 25 mM *S*-methyl methanethiosulfonate (MMTS) with a 2 M stock prepared in dimethylformamide (DMF)) and incubated for 20 min at 50°C to block free cysteine residues. Acetone precipitation was performed to remove excess MMTS, and nitrosothiols were reduced by 12.5 mM ascorbic acid to permit reaction with the sulfhydryl-specific biotinylation reagent HPDP-biotin (Thermo Fisher Scientific, 21341). Biotinylated proteins were pulled down using streptavidin agarose beads (Thermo Fisher Scientific, 20353), and western blot analysis was performed.

2.8 LC-MS/MS

HEK293T cells were treated with 200 μM SNOC for 30 min. Total cell lysates were then subjected to biotin-switch assay. Pull-down samples were treated with 0.25 μg/mL trypsin (Promega) overnight at 37°C, and the digested peptides were cleaned-up using self-made C18 and SCX (3M Empore solid phase extraction disk) stage tips. The sample was subjected to liquid chromatography using an EASY-nLC 1000 instrument (Thermo Fisher Scientific) coupled with a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (MS, Thermo Fisher Scientific) with a nanospray ion source in positive mode. The processed peptides were separated using a nano-HPLC C18 capillary column (0.075 × 150 mm, 3 μm) (Nikkyo Technos). A 60-min gradient was used at a flow rate of 300 nL/min, including 5–35% Bin over 48 min and then 35–65% Bin over 12 min (solvent A, 0.1% formic acid; solvent B, 100% CH3CN, 0.1% formic acid). The resulting MS and MS/MS data were used to search the Swiss Prot database using

Proteome Discoverer (Thermo Fisher Scientific) with MASCOT search engine software (Matrix Science).

2.9 *In vitro* ubiquitination assay

The CHIP/Luciferase Ubiquitination Kit and human recombinant UBE2D1 were used to detect the direct effect of NO on UBE2D1 enzyme activity. An *in vitro* ubiquitination assay was carried out according to the manufacturer's instructions except for enzyme pre-treatment. Specifically, we pre-treated 1 μM UBE2D1 with SNOC (at 1, 10, or 50 μM) or decayed (old) SNOC (50 μM) in the dark for 2 hr at room temperature.

2.10 Statistical analysis

All experiments were repeated independently for a total of at least three times. All data are presented as the mean ± standard error of the mean (S.E.M.). Statistical comparisons were carried out using two-tailed non-paired Student's t-tests or one-way ANOVA with post hoc Bonferroni's test using Graphpad Prism 8 software (Graphpad Software). p values of <0.05 were considered significant.

3. Results

3.1 NO inhibits the ubiquitination of SGK1, ERAD substrate.

We and other groups have reported that molecular chaperones and ER stress sensors are targets for NO [8, 10, 11, 14]; however, the effect of NO on ERAD remains largely unknown. To explore this issue, we focused on SGK1, a well-known ERAD substrate that has a short half-life (about 30 min) [15].

To confirm whether SGK1 is degraded by the ubiquitin-proteasome system, we

transiently transfected HEK293T cells with a SGK1 and treated the resulting cells with a proteasome inhibitor (MG132) or with an autophagy inhibitor (LY294002). We confirmed the phosphoinositide-3 kinase (PI3K) inhibitory effect of LY294002 by monitoring Akt phosphorylation. As expected, MG132, but not LY294002, inhibited the degradation of SGK1 (**Fig. 1A**).

To monitor whether nitrosative stress affects the ubiquitination of SGK1, we co-transfected HEK293T cells with both Myc-SGK1 and T7-ubiquitin and treated the resulting cells with either of two different physiological NO donors, *S*-nitrosocysteine (SNOC) or *S*-nitrosoglutathione (GSNO). Lysates of the treated cells then were subjected to immunoprecipitation to purify SGK1, and the resulting protein was assessed for ubiquitination. Probing of the western blots with anti-Myc antibody revealed the presence of three distinct non-ubiquitinated protein bonds. We speculated that these three bands correspond to post-translationally modified versions of SGK1; notably, previous work has identified several potential phosphorylation sites within SGK1 [16]. Interestingly, we found that polyubiquitination of SGK1 was significantly decreased by both SNOC and GSNO treatments **(Fig. 1B** and **C,** respectively). These results indicated that nitrosative stress has an inhibitory effect on the ubiquitination of SGK1.

3.2. Identification of Cys85 of UBE2D1 as the target site of *S*-nitrosylation.

Given that NO regulates the enzymatic activity of proteins via *S*-nitrosylation, a post-translational modification [8, 10, 11, 14, 17], we hypothesized that NO similarly would modulate components of the ERAD. To test this prediction, we subjected SNOC-treated HEK293T cells lysates to a biotin-switch assay, which permits biotinylation of

S-nitrosylated moieties [18], and used the biotin label to purify the *S*-nitrosylated proteins. We analyzed the resulting proteins using LC-MS/MS. This analysis identified UBE2D1, a known component of the ubiquitin-proteasome system [19, 20], as a novel target for modification by NO. Notably, the MS/MS spectrum of the y6 peptide indicated a mass shift (+428) consistent with the biotin to the cysteine residue of the y5 peptide, indicating that Cys85 of UBE2D1 is the residue targeted by *S*-nitrosylation (**Fig. 2A**). UBE2D1 plays an important role as an upstream regulator of CHIP, which itself regulates ERAD by serving as an E3 ubiquitin ligase. Also, this system is involved in the degradation of SGK1 [19–23]. The previously published 3D structure of the interaction between ubiquitin and UBE2D1 (PDB:3PTF, https://www.rcsb.org/, [24]) is shown in **Fig. 2B.** In the figure, the red circle shows the non-covalent bond between ubiquitin and Cys85 of UBE2D1, suggesting that this region is important for this enzyme's activity. To further confirm *S*-nitrosylation of UBE2D1, we used SNOC treated HEK293T cells overproducing FLAG-UBE2D1. Further biotin-switch analysis demonstrated that UBE2D1 indeed undergoes *S*-nitrosylation in a NO-donor-dose-dependent manner (**Fig. 2C, D)**.

Based on these lines of evidence, we hypothesized that Cys85 of UBE2D1 is a target site for NO, and that *S*-nitrosylation of this site may decrease the ubiquitination of substrates, thereby downregulating the ERAD pathway.

3.3 NO donor treatment attenuates the ubiquitin-conjugating activity of UBE2D1

To test our hypothesis, we performed an *in vitro* UBE2D1 ubiquitin-conjugating activity assay using luciferase as the target protein to assess the effect of pretreating recombinant UBE2D1 with SNOC (**Fig. 3A**). Following the reaction, we tested the

assay products by immunoblotting with anti-ubiquitin (**Fig. 3B**) or anti-luciferase antibodies (**Fig. 3C**) to detect the ubiquitination of the substrate (luciferase). Intriguingly, we found that pre-treatment with SNOC inhibited the ubiquitination of luciferase in a dose-dependent manner (**Fig. 3B, 3C**). Figure **3D** shows the quantification analysis of the gel shown in **Fig. 3C**. To measure UBE2D1's ubiquitination efficacy, we quantified high-molecular-weight (> 70-kDa) signals, that is, products that exceeded the predicted size of monoubiquitinated luciferase. The data showed that the ubiquitination activity of UBE2D1 was significantly decreased by pretreatment with SNOC at 10 and 50 μM.

4. Discussion

Several studies have indicated that the NO is an important regulator of various cellular processes [5–7]. However, excess NO levels can cause various diseases, including neurodegenerative diseases, cancer, and diabetes [8-10]. Notably, both the accumulation of the unfolded proteins and excessive amounts of NO are observed in the brains of patients with neurodegenerative diseases [25,26]. In previous work, we demonstrated that nitrosative stress exacerbates ER stress via *S*-nitrosylation of the molecular chaperone PDI [8] and the ER stress sensor IRE1 [11].

In the present study, we showed that NO attenuates the ubiquitination of ERAD substrates (**Fig. 1, 3**). Additionally, we identified UBE2D1 as a novel *S*-nitrosylated protein. Specifically, we observed that UBE2D1 is *S*-nitrosylated at an active site residue, Cys85, a modification that is exposed to impair UBE2D1's ubiquitin-binding activity (**Fig. 2**). Intriguingly, an examination of screening data for *S*-nitrosylated proteins reported by another group indicates that various E2 enzymes are similarly *S*-

nitrosylated at their enzymatic active sites [27]. Additionally, UBE2D3, which belongs to the same family as UBE2D1, has been shown to be *S*-nitrosylated, a modification that decreases UBE2D3's activation of chaperone-mediated LAMP2a-dependent autophagy [28]. In other work, we demonstrated that NO exacerbates ER stress via S-nitrosylation of the molecular chaperone PDI [8] and of the ER stress sensor IRE1 [11]. Collectively, these results indicate that many branches of responses against ER stress are weakened by nitrosative stress, prolonging ER stress by potentiating the accumulation of the unfolded proteins.

Although further analysis is required, it is tempting to hypothesize that UBE2D1 may transnitrosylate CHIP to propagate NO's effect on ERAD. Transnitrosylation is a serial modification system that permits the transfer of NO from a given protein to other protein members of the same complex. As a result, transnitrosylation extends the range of processes controlled by SNO modification. Interestingly, CHIP, which is an E3 ligase of ERAD and functions downstream of UBE2D1, also is known to be *S*-nitrosylated [29]. In a chain reaction, E3 ligase activity might be attenuated to halt ERAD. Indeed, other groups have reported transnitrosylation [30, 31].

Our results show that nitrosative stress decreases the level of ubiquitinated protein. This observation contrasts with previous reports that the level of ubiquitinated proteins is increased in the brains of patients with neurodegenerative diseases such as PD [32], and AD [33]. However, one early-onset variant of PD, autosomal recessive PD (ARPD), results from Parkin loss-of-function mutations but is not associated with the accumulation of ubiquitin-positive aggregates in the brains of patients [34]. Other work has suggested that the formation of ubiquitin-positive aggresomes may play a protective role [35]. Although ubiquitylome analysis using the brains of AD patients revealed that

the ubiquitination levels of many proteins were increased, that report also showed that the level of the ubiquitination was decreased in certain proteins [35]. SGK1 might be one such protein. SGK1 phosphorylates tau at Ser214 [36], and this modification is correlated with the formation of paired helical filaments [37]. In this present study, we showed the ubiquitination of SGK1 is downregulated by nitrosative stress (Fig. 1); it is tempting to propose that nitrosative stress contributes to abnormal tau phosphorylation by leading to the accumulation of SGK1.

Further analysis will be needed to investigate whether SNO-UBE2D1 contributes to the accumulation of ERAD substrates. Nonetheless, our data suggested that NO-mediated ERAD inhibition results in an increase in the level of non-ubiquitinated unfolded proteins, a known mediator of neurotoxicity.

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Figure legends

Figure 1. Effect of NO on the SGK1, ERAD substrate.

A: HEK293T cells were transfected with Myc-SGK1 and incubated for 24 hr. Subsequently, the cells were treated with MG132 or LY294002 for 3 hr. **B, C:** (Upper) Myc-SGK1, T7-ubiquitin, and FLAG-UBE2D1 were co-expressed in HEK293T cells. At 24 hr after transfection, cells were co-treated with MG132 (25 μM) and SNOC (200 μM, 2 hr) or GSNO (200 μM, 3 hr). The control sample was treated with decayed (old) SNOC or GSH (reduced form of glutathione). Cell lysates were immunoprecipitated with anti-Myc antibody and subjected to western blotting with indicated antibodies. (Lower) The level of ubiquitinated SGK1 (indicated as [Ub]n-SGK1) was quantified within each sample and normalized to the level of non-ubiquitinated SGK1 within the respective sample. Data were analyzed using a two-tailed non-paired Student's t-test. Values are expressed as the mean ±S.E.M. (n=3); ** $p<0.01$ compared to treatment with old SNOC or GSH.

Figure. 2. Identification of the *S*-nitrosylated ubiquitin-conjugating enzyme involved in ERAD.

A: HEK293T cells were exposed to SNOC (200 μM) for 30 min, and cell lysates were subjected to LC-MS/MS in conjunction with biotin-switch assays. MS/MS spectra yieled m/z peaks corresponding to UBE2D1 peptide fragments containing biotinylated Cys85. This analysis showed that UBE2D1 is *S*-nitrosylated at Cys85 by a physiological NO donor. **B:** The 3D structure of the complex of UBE2D1 and ubiquitin (PDB: 3PTF). The red circle show that ubiquitin binds to Cys85 of UBE2D1. **C:** HEK293T cells were transfected with FLAG-UBE2D1. At 24 hr after transfection, cells were treated with the

indicated concentrations of SNOC for 30 min. Cell lysates were subjected to a biotin-switch assay to detect SNO-UBE2D1. **D:** The relative intensity of SNO-UBE2D1 in panel **C** was quantified and normalized to input UBE2D1. Data represent the fold increase of SNO-UBE2D1 in SNOC-treated cells compared to that in old-SNOC-treated cells using one-way ANOVA with post hoc Bonferroni's test. Values are expressed as the mean ± S.E.M. (n=3); ** $p<0.01$ versus sample treated with old SNOC.

Figure. 3. Treatments with NO donor attenuates the enzymatic activity of UBE2D1. **A:** A flow chart of the *in vitro* ubiquitination assay. UBE2D1 was pretreated with indicated concentrations of SNOC for 2 hr to promote formation of *S*-nitrosylated UBE2D1. **B, C:** Detection of ubiquitinated luciferase using anti-ubiquitin and anti-luciferase antibodies. **D:** The level of ubiquitinated luciferase (indicated as [Ub]n) was quantified and normalized to the level in the control sample using one-way ANOVA with post hoc Bonferroni's test. Data are expressed as the mean ±S.E.M. (n=4); ** $p<0.01$, * $p<0.05$.

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